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Six N-alkyl and N-aryl 5-(1,3,3-trimethylindolinyl) carbamates were synthesized and studied for their structure-activity relationships in inhibiting eel acetylcholinesterase (AChE). The carbamates were 5-(1,3,3-trimethylindolinyl)N,N-dimethylcarbamate (Cui Xing Ning) (I), 5-(1,3,3-trimethylindolinyl)N-methylcarbamate (II), 5-(1,3,3-trimethylindolinyl)N-ethylcarbamate (III), 5-(1,3,3-trimethylindolinyl)N,N-diethylcarbamate (IV), 5-(1,3,3-trimethylindolinyl)N-heptylcarbamate (V), and 5-(1,3,3-trimethylindolinyl) N-(3-cholorophenyl)carbamate (VI). The inhibition studies were carried out at 25.0°C at pH 7.60. The rank order of the k_i values for eel AChE inhibition is II > V > I > III > VI > IV. Compound II has a greater affinity for the enzyme than any irreversible inhibitor cited in the literature (K_i = 7.14 × 10^{-8}M). Our findings should aid in the application of these carbamates (1) for counteracting the cholinergic problems associated with various diseases, and (2) for developing potential pretreatment compounds for organophosphate poisoning

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ANTICHOLINESTERASE ACTIVITY OF POTENTIAL THERAPEUTIC 5-(1,3,3-TRIMETHYLINDOLINYL) CARBAMATES†

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(*Received 1 February, 1990*)

Six N-alkyl and N-aryl 5-(1,3,3-trimethylindolinyl) carbamates were synthesized and studied for their structure-activity relationships in inhibiting eel acetylcholinesterase (AChE). The carbamates were 5-(1,3,3,-trimethylindolinyl)N,N-dimethylcarbamate (Cui Xing Ning) (**I**), 5-(1,3,3-trimethylindolinyl)N-methylcarbamate (**II**), 5-(1,3,3-trimethylindolinyl)N-ethylcarbamate (**III**), 5-(1,3,3-trimethylindolinyl)N,N-diethylcarbamate (**IV**), 5-(1,3,3-trimethylindolinyl)N-heptylcarbamate (**V**), and 5-(1,3,3-trimethylindolinyl) N-(3-cholorophenyl)carbamate (**VI**). The inhibition studies were carried out at 25.0°C at pH 7.60. The rank order of the k_i values for eel AChE inhibition is **II** > **V** > **I** > **III** > **VI** > **IV**. Compound **II** has a greater affinity for the enzyme than any irreversible inhibitor cited in the literature ($K_d = 7.14 \times 10^{-8}$M). Our findings should aid in the application of these carbamates (1) for counteracting the cholinergic problems associated with various diseases, and (2) for developing potential pretreatment compounds for organophosphate poisoning.

KEY WORDS: Acetylcholinesterase, carbamates, inhibition.

INTRODUCTION

A limited number of carbamates have been found useful in counteracting the cholinergic problems associated with Alzheimer's disease, Huntington's disease, tardive dyskinesia, myasthenia gravis, and as pretreatment compounds for organophosphorus poisoning (pesticides and nerve agents). Two of the carbamates most frequently used are pyridostigmine and physostigmine. Both of these compounds are inhibitors of acetylcholinesterase through carbamylation of the esteratic site of the enzyme.[1] This inhibition is subsequently reversed upon decarbamylation.[2] In their application as cholinesterase antagonists, pyridostigmine and physostigmine exhibit a number of desirable similarities. However, each has specific undesirable characteristics. Pyridostigmine has a relatively short duration of action and does not penetrate the blood brain barrier. Some of the less desirable characteristics of physostigmine include its high toxicity, narrow therapeutic window, short duration of action, and other ancillary side effects. In our exploration to find carbamates devoid of some of these negative characteristics, we have prepared analogs of 5-(1,3,3-trimethylindolinyl)N,N-dimethylcarbamate (**I**). Compound **I**, which has structural similarities to physostigmine, was first prepared by Ahmed and Robinson in 1965.[3] Since that time the only references

†The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Department of Defense.

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to it are found in the Chinese literature, where it is frequently referred to as Cui Xing Ning.[4] In this paper we report the eel acetylcholinesterase (AChE) inhibition constants of **I** and of five of its analogs. The synthesis of these analogs, in which the dimethylcarbamyl group of **I** has been substituted with N-methylcarbamyl (**II**), N-ethylcarbamyl (**III**), N,N-diethylcarbamyl (**IV**), N-heptylcarbamyl (**V**), and N-(3-cholorophenyl)carbamyl (**VI**), are described. Cholinesterase inhibition constants are among the initial parameters for the identification of therapeutically functional cholinesterase antagonists. For comparison purposes we have included our results with those for physostigmine (**VII**) and pyridostigmine (**VIII**).

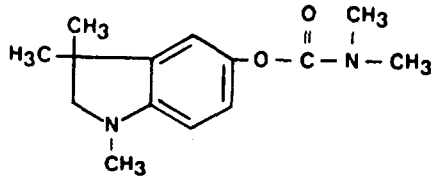
MATERIALS AND METHODS

Synthesis (General)

The parent compound (**I**) and the five new carbamates (**II–VI**) used in our inhibition studies were all prepared from the intermediate 5-hydroxy-1,3,3-trimethylindoline by treatment with a substituted carbamyl chloride (**I** and **II**) or the appropriate isocyanate (**III–VI**). The common intermediate was synthesized by literature procedures[5,9,10] with several modifications to improve yields or facilitate handling. Our first major modification was in the conversion of 1,3-dimethyl-5-hydroxyindolin-2-one to 5-methoxy-1,3,3-trimethylindolin-2-one by methylation of the hydroxy compound using successive treatment with sodium hydride and methyl iodide in dimethylformamide. The second major modification was in the reduction of the resulting keto compound to 5-methoxy-1,3,3-trimethylindoline using lithium aluminium hydride. This reduction has been reported for an analogous indolin-2-one.[17] Three of the carbamates (**I**, **IV**, and **V**) were oils and were isolated as the hydrochloride salt (**I**) or as the hydrobromide salt (**IV** and **V**). The structures of the free bases of **I–VI** are included in Figure 1.

5-(1,3,3-Trimethylindolinyl)N,N-dimethylcarbamate Hydrochloride (**I**). This carbamate, mp 190–192°C was prepared by Chemsyn Science Laboratories[11] following published procedures.[5,9,10]

5-(1,3,3-Trimethylindolinyl)N-methylcarbamate (**II**). To a stirred ice-cold solution of 5-hydroxy-1,3,3-trimethylindoline hydrobromide (23 g, 89.1 mmol) in water (200 ml) covered with ether (300 ml) was added sodium carbonate (4.5 g, 42.6 mmol). The mixture was stirred *ca.* 5 min and the layers were separated. Additional sodium carbonate (4.5 g, 42.6 mmol) was added to the water layer which was extracted again with ether (2 × 300 ml). The combined organic layer was washed with water (2 × 300 ml) and dried ($MgSO_4$). The solvent was removed under reduced pressure (aspirator) and replaced with benzene (300 ml). This solution of the free base in benzene was stirred at ambient temperature, under a nitrogen atmosphere, with methyl isocyanate (10.2 g, 178.2 mmol) and sodium metal (*ca.* 30 mg) for 24 h. The mixture was filtered and the excess methyl isocyanate was removed at room temperature under reduced pressure (aspirator). The solution was concentrated (aspirator, 35–40°C) to yield a light pink solid (20.4 g). This material was chromatographed (silica gel, 250 g, 5.5 cm × 31 cm column), eluting with chloroform/ether (4:1), to give a white solid. Recrystallization from a mixture of tetrahydrofuran (60 ml) and *n*-hexane (300 ml) gave 18.9 g (90%) of pure title compound as white crystals.



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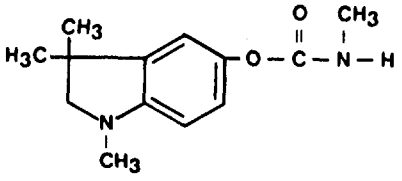

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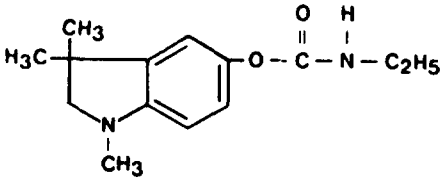

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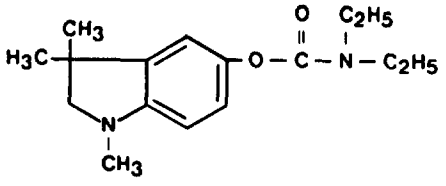

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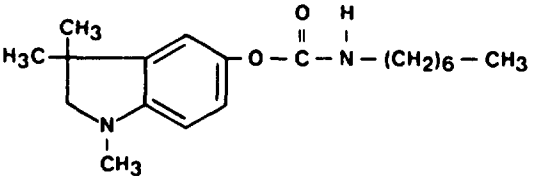

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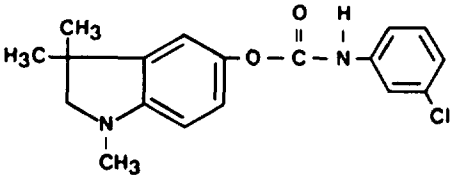

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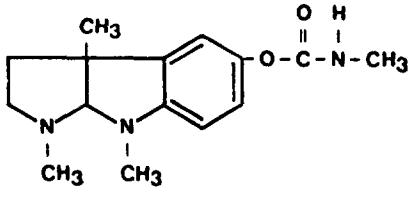

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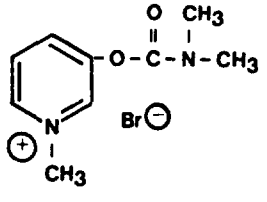

VIII

*I = 5-(1,3,3-TRIMETHYLINDOLINYL)N,N-DIMETHYLCARBAMATE
II = 5-(1,3,3-TRIMETHYLINDOLINYL)N-METHYLCARBAMATE
III = 5-(1,3,3-TRIMETHYLINDOLINYL)N-ETHYLCARBAMATE
IV = 5-(1,3,3-TRIMETHYLINDOLINYL)N,N-DIETHYLCARBAMATE
V = 5-(1,3,3-TRIMETHYLINDOLINYL)N-HEPTYLCARBAMATE
VI = 5-(1,3,3-TRIMETHYLINDOLINYL)N-(3-CHLOROPHENYL)CARBAMATE
VII = PHYSOSTIGMINE
VIII = PYRIDOSTIGMINE BROMIDE

FIGURE I Chemical structures of carbamates I through VIII.

mp 141–143°C (lit.,[10] 144.5–145°C). *Anal.* Calcd. for $C_{13}H_{18}N_2O_2$ (234.29): C, 66.64; H, 7.74; N, 11.96%. Found: C, 66.83; H, 7.92; N, 12.10%. The IR and NMR spectra were in agreement with the assigned structure.

5-(1,3,3-Trimethylindolinyl)N-ethylcarbamate (**III**). 5-Hydroxy-1,3,3-trimethylindoline hydrobromide (21 g, 81.3 mmol) was converted to the free base as described for compound (**II**). A solution of the free base in benzene (300 ml) was stirred at ambient temperature under a nitrogen atmosphere and treated with ethyl isocyanate (12.1 g, 170.4 mmol) and a tiny piece of sodium metal (*ca.* 30 mg). After 24 h the mixture was filtered and the filtrate was evaporated (aspirator) to give 20.9 g (98.6%) of product as a light pink solid. The solid was purified by column chromatography (silica gel, 250 g), eluting with chloroform/ether (4 : 1), to give a white solid. Recrystallization from a mixture of tetrahydrofuran (40 ml) and *n*-hexane (240 ml) yielded 15.6 g (73.6%) of pure title compound as white crystals, mp 105–107°C. *Anal.* Calcd. for $C_{14}H_{20}N_2O_2$ (248.32): C, 67.71; H, 8.12; N, 11.28%. Found: C, 67.86; H, 7.90; N, 11.30%. The IR and NMR spectra were in agreement with the assigned structure.

5-(1,3,3-Trimethylindolinyl)N,N-diethylcarbamate Hydrobromide (**IV**). To a mixture of 5-hydroxy-1,3,3-trimethylindoline hydrobromide (22 g, 85.2 mmol), tetrabutylammonium iodide (880 mg), and diethylcarbamyl chloride (27.7 g, 204.5 mmol) in benzene (195 ml) was added dropwise 50% aqueous sodium hydroxide (44 g, 550 mmol). The mixture was heated at reflux for 1 h. After cooling, water (100 ml) was added. The water layer was separated and extracted with benzene (2 × 100 ml). The combined benzene layer was washed with brine (2 × 100 ml), dried ($MgSO_4$), and evaporated (aspirator). The residue was purified by chromatography twice over silica gel (1 × 480 g, 1 × 300 g), eluting with chloroform/ethyl acetate (9 : 1). The product-containing fractions were combined and concentrated (aspirator) to give 23.1 g (98%) of the free base of **IV** as a light brown liquid. A portion of this material (21.9 g) was dissolved in anhydrous ether (500 ml), cooled in an ice bath, and hydrogen bromide gas was passed through the solution. The creamy solid thus obtained was recrystallized twice from a mixture of isopropyl alcohol and ether to give 22.5 g (78%) of pure product as white crystals, mp 169.5–171.5°C (dec). *Anal.* Calcd for $C_{16}H_{24}N_2O_2 \cdot HBr$ (357.30): C, 53.79; H, 7.05; Br, 22.36; N, 7.84%. Found: C, 53.96; H, 6.79; Br, 22.55; N, 7.76%. The IR and NMR spectra were in agreement with the assigned structure.

5-(1,3,3-Trimethylindolinyl)N-heptylcarbamate Hydrobromide (**V**). 5-Hydroxy-1,3,3-trimethylindoline hydrobromide (21 g, 81.3 mmol) was converted to the free base as described for compound **II**. A solution of the free base in benzene (300 ml) was stirred at ambient temperature, under a nitrogen atmosphere, with *n*-heptyl isocyanate (11.49 g, 81.34 mmol) and sodium metal (*ca.* 30 mg) for 6 days. It was then heated at reflux for 30 h. After cooling, the mixture was filtered and the solvent was removed under reduced pressure (aspirator) to give a light brown liquid which was chromatographed (silica gel, 250 g, 5.5 cm × 31 cm column) with chloroform/ether (4 : 1). The product-containing fractions were combined and concentrated (aspirator) to give a light yellow solid. The liquid was dissolved in anhydrous ether (500 ml), cooled in an ice bath, and anhydrous hydrogen bromide gas bubbled through the solution. The cream-colored solid that precipitated was collected and recrystallized from a mixture of tetrahydrofuran (180 ml) and petroleum ether (300 ml) to give white crystals (23.8 g). Two additional recrystallizations from a mixture of tetrahydrofuran (165 ml)

and petroleum ether (270 ml) yielded 22.8 g (70%) of pure title compound, mp 94–96°C. *Anal.* Calcd for $C_{19}H_{30}N_2O_2 \cdot HBr$ (399.38): C, 57.14; H, 7.82; Br, 20.01; N, 7.01%. Found: C, 56.98; H, 7.72; Br, 19.86; N, 6.95%. The IR and NMR spectra were in agreement with the assigned structure.

Physostigmine (**VII**) *and Pyridostigmine* (**VIII**). Physostigmine (salicylate salt), Lot No. NDA-22120, was obtained from O'Neal, Jones, and Feldman, St. Louis, MO 63043. Pyridostigmine (bromide salt), Lot. No. 426111, was obtained from Hoffman-LaRoche Inc., Nutley, NJ 07110.

Biochemistry and Pharmacology

Preparation of Buffer Solution. The pH 7.60 MOPS [3-(N-morpholino)propanesulfonic acid] buffer was 0.10 M in the buffering component and contained 0.01 M Mg^{2+} ($MgCl_2$), 0.002% sodium azide, and 0.01% bovine serum albumin. The pH value was determined at 25.0°C using a Beckman Model 4500 digital pH meter.

Eel Acetylcholinesterase. Eel acetylcholinesterase (Eel AChE, EC 3.1.1.7) was purchased from Sigma Chemical Company. It was obtained as a lyophilized powder, 1130 units/mg solid, containing approximately 15% buffer salts. Enzyme concentrate was prepared by dissolving 4.5 mg of this powder in 0.80 ml of a previously boiled solution containing 0.225 M KCl, 0.10% gelatin, and 0.02% soldium azide. It was stored in a refrigerator at 4°C. Enzyme stock solutions for inhibition studies were prepared daily by adding 7.50 μl of the enzyme concentrate to 125.0 ml of the pH 7.60 MOPS buffer.

Inhibition of Eel Acetylcholinesterase by **I–VIII.** In a typical inhibition experiment enzyme stock solution at 25.0°C was dispensed into four 25.0 ml volumetric flasks. To determine the pre-inhibition activity of the enzyme the remaining enzyme stock solution was assayed spectrophotometrically (in triplicate) at 272.5 nm using a Beckman DU70 instrument with kinetic analysis software. The substrate for 1.00 ml aliquots of enzyme stock solution was 10.0 μl of 0.39 M phenyl acetate in acetonitrile. Assay concentration of the phenyl acetate substrate equals 3.9×10^{-3} M. A typical activity for an enzyme solution prepared as described was 0.310 absorbance units/min (SD = 0.002). Appropriate stock solutions of the selected carbamate inhibitor were prepared in distilled water or acetonitrile. Cuvettes were allowed to equilibrate in the cell holder (25.0°C) of the spectrophotometer. At time zero 25.0 μl of the carbamate inhibitor solution was added to a 25.0 ml volumetric flask containing enzyme stock solution (25.0°C). At 3.0, 6.0, 9.0, 12.0, and 15.0 min, 1.00 ml aliquots of enzyme/inhibitor solution were assayed spectrophotometrically using phenyl acetate for the substrate.

Evaluation of Eel AChE Inhibition Constants (k_1, k_2, and K_d). The kinetic constants of AChE inhibition were determined by the method of Main and co-workers.[12-14] Quadruplicate determinations of residual rates versus enzyme-inhibitor incubation times (3.0, 6.0, 9.0, 12.0, and 15.0 min) were made for each carbamate at four different concentrations. The resultant activity versus time plots were evaluated by the program NKINETICS[15] to give the corresponding values for k_{obsd}, the apparent first order rate constant. The k_{obsd} and the corresponding inhibitor concentration are the basic

paramaters for the calculation of the enzyme inhibition constants. The inhibition constants are evaluated from a plot of $1/k_{obsd}$ versus 1/[Inhibitor]. The bimolecular inhibition constant, k_i is the reciprocal of the slope, the unimolecular carbamylation constant, k_2, is the reciprocal of the intercept, and the equilibrium dissociation constant, K_d, $= k_2/k_i$. In our studies one run from each of the quadruplicate determinations at the different inhibitor concentrations was used to evaluated K_i, k_2, and K_d. The average value and the corresponding standard deviation for each of the four determinations for **I-VIII** are shown in Table I.

Acute Intramuscular and Oral Toxicity (Mice) of **I, VII,** *and* **VIII.** Twenty-four hour LD_{50} estimates were obtained by treating mice intramuscularly or orally with **I, VII,** and **VIII** (6 doses × 5 animals/dose). Intramuscular doses were given by injection in a hind limb in a dose volume of 0.5 ml/kg. The oral doses were given by gavage in a dose volume of 10.0 ml/kg. The LD_{50} estimates and 95% confidence limits were determined by the method of Bliss.[16] The results of these studies are shown in Table II.

TABLE I
Kinetic constants for the inhibition of eel acetylcholinesterase, 0.10 M MOPS buffer, pH 7.60, 25.0°C

Compound	K_d (M)	k_i ($M^{-1}min^{-1}$)	k_2 (min^{-1})
I	3.47×10^{-7} (0.52)†	2.88×10^{5} (0.23)	0.01 (0.01)
II	7.14×10^{-8} (2.43)	3.41×10^{6} (0.18)	0.24 (0.07)
III	2.54×10^{-6} (1.20)	2.81×10^{5} (0.07)	0.71 (0.32)
IV	1.31×10^{-5} (0.34)	3.88×10^{4} (0.01)	0.05 (0.01)
V	1.66×10^{-6} (0.54)	4.08×10^{5} (0.20)	0.67 (0.19)
VI	3.69×10^{-6} (1.18)	9.37×10^{4} (0.15)	0.35 (0.11)
VII	3.14×10^{-7} (2.68)	2.14×10^{6} (0.34)	0.31 (0.12)
VIII	5.50×10^{-6} (1.39)	3.42×10^{4} (0.29)	0.18 (0.04)

† = Standard Deviation of $n = 4$.

TABLE II
Acute toxicity of selected carbamates in mice†

Carbamate	LD_{50} (mg/kg) Intramuscular	Oral
Cui Xing Ning Hydrochloride (I)	5.25 (4.32–6.45)‡	15.30 (7.03–18.82)
Physostigmine Salicylate (VII)	0.69 (0.47–1.06)	3.89 (3.48–4.35)
Pyridostigmine Bromide (VIII)	3.07 (2.30–4.10)	26.17 (22.59–30.38)

†In conducting the research described in this report, the Investigators adhered to the "Guide for the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council."

‡95% Confidence Interval.

RESULTS AND DISCUSSION

New carbamates related to physostigmine are of interest because of their potential application in various therapeutic regimens. Many members of the carbamate class of compounds are biologically active because of their structural complementarity to the active site of various cholinesterases. Covalent bond formation in the inhibition of AChE by carbamates involves carbamylation of the active site serine. This carbamylation proceeds by the mechanisms shown in Eq. (1).[18]

$$\text{AChE} + \text{C–X} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{AChE·C–X} \xrightarrow{k_2} \text{AChE–C} + \text{X} \qquad (1)$$

The characteristics of such an inhibitor are expressed in terms of its dissociation constant, K_d (i.e., k_{-1}/k_1), and the subsequent unimolecular bonding constant, k_2, to form the inhibited enzyme, AChE–C. An overall rate of inhibition is commonly stated in terms of a bimolecular reaction constant, k_i (i.e., k_2/K_d). The results from our inhibition experiments with the carbamates **I–VIII** are shown in Table I.

In the carbamylation of cholinesterases there are a variety of contributing factors. These include inductive effects, resonance effects, hydrophobicity, steric factors, charged groups, and chirality. The limited number of carbamates in Table I precludes identifying quantitatively the role that each factor contributes to our results. However, our findings are in basic accord with recent publications of similar studies using physostigmine analogs.[19–22] The analogies and significant differences merit comment.

In our studies, as well as in those of Yu,[20] Bores,[21] and Atack,[22] it was found that extension of the carbamyl side chain via methylene groups does not greatly reduce the ability of the carbamyl group to interact with the esteratic site of the enzyme (compare **II**, **III**, and **V** in Table I). From a drug development point of view, the ability to modify lipophilicity without severely diminishing the acetylcholinesterase activity substantially enhances the prospect of maximizing a favorable distribution of the drug with the desired pharmacokinetics.

Steric limitations, as reflected in our results from those carbamates that have only one substituent group on the carbamyl nitrogen, are relatively minor. As is evident from Table I, the N-heptyl analogue (**V**) and the N-(3-chlorophenyl) analogue (**VI**) are approximately equal to the ethyl analogue (**III**). Each of these analogues is only one order of magnitude below the results for the N-methyl compound (**II**). This is in contrast with the early results published by Kolbezen in 1954.[23] He and his coworkers examined a series of N-methyl, N-ethyl, N-benzyl, and N-phenyl carbamates. With the exception of the N-methyl derivatives, they found that the other compounds produced little or no carbamylation of fly brain acetylcholinesterase. These early results might have contributed to the development of carbamate insecticides emphasizing the N-methyl group (i.e., Aldicarb, Baygon, Carbaryl, Methomyl, etc.).

The most significant apparent steric effects we observed resulted from the introduction of a second substituent on the carbamyl nitrogen atom. Based on k_i, our dimethyl compound (**I**) is approximately 100 times less potent than the monomethyl compound (**II**). Though Brossi[19] reported in 1986 that dimethylphysostigmine was a more potent inhibitor of eel AChE than physostigmine (a N-methylcarbamate), that observation has not been supported by subsequent investigators.[20,22] More striking are the results with the diethyl compound (**IV**) and the ethyl compound (**III**). Based on k_i, the diethyl compound (**IV**) is approximately 7000 times less potent than the monoethyl compound (**III**). The very significant decrease in k_i we observed with **IV** is in agreement with similar findings by Bores[21] and Atack.[22]

It is of interest to note that these carbamate results are inconsistent with the inhibition results reported for several different classes of organophosphorus compounds. In studies with a variety of 4-nitrophenyl dialkyl phosphates Ooms found that the diethyl compound was a more potent inhibitor of acetylcholinesterase, butyrylcholinesterase, chymotrypsin, and trypsin than the dimethyl compound.[24,25] Ooms also reported that diethyl phosphorofluoridate was a more potent inhibitor of these enzymes than dimethyl phosphorofluoridate.

One final point relative to the data in Table I. The K_d value of 7.14×10^{-8} M for **II** is the smallest value that has been reported for this constant for any inhibitor of acetylcholinesterase which produces a covalent bond between the inhibitor and the enzyme. This includes the very reactive and toxic organophosphorus compounds of military significance. At pH 7.0 in 0.067 M phosphate buffer the K_d value for isopropyl methylphosphonofluoridate (sarin) is 2.2×10^{-6} M and the same value[26] for pinacolyl methylphosphonofluoridate (soman) is 4.0×10^{-5} M. The strong affinity of **II** for the enzyme may make this compound, or closely related derivatives, attractive for certain pharmacological applications.

Intrinsic to the development of 5-(1,3,3-trimethylindolinyl)carbamates as drugs to counteract various cholinergic problems is some assurance that they will have a safe therapeutic index. Our first results for the acute toxicity of Cui Xing Ning (**I**), in mice, are given in Table II. Comparable data for pyridostigmine and physostigmine, both of which are Food and Drug Administration approved drugs, are included for comparison. As is evident in Table II, **I** was 1.6 and 9.4 times *less* toxic than pyridostigmine and physostigmine, respectively, following intramuscular administration. Orally **I** was 1.7 times *more* toxic than pyridostigmine, but 3.9 times *less* toxic than physostigmine. These findings are basically in parallel with the statement by Ji and Wei[5] that **I** has a "low toxicity". Total results to date suggest that the toxicity of **I** and other tailored analogues should not be a deterrent in replacing currently used carbamates in various therapeutic regimens.

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